

REPORT DOCUMENTATION PAGE

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE (DD-MM-YYYY) 27-08-2008		2. REPORT TYPE Final		3. DATES COVERED (From - To) 01/03/2007-31/05/2008	
4. TITLE AND SUBTITLE Development of New Genetic Manipulation Tools For Metabolic Engineering of Diatoms				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER FA95550-07-1-0386	
				5c. PROGRAM ELEMENT NUMBER	
				5d. PROJECT NUMBER	
6. AUTHOR(S) Mark Hildebrand				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California, San Diego 9500 Gilman Dr. La Jolla, CA 92093-0202				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Air Force Office of Scientific Research 875 N. Randolph St. Suite 324, Room 3112 Arlington VA 22203-1768 <i>Dr Walter Kozumbo/NL</i>				10. SPONSOR/MONITOR'S ACRONYM(S) AFOSR	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT dist a					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT This project's goal was to develop genetic manipulation tools for metabolic engineering of diatoms for biodiesel lipid production and other purposes. New diatom selectable markers were tested, relying on mutations to antibiotic resistance in two ribosomal genes. Three new diatom transformation vectors were constructed, using the nat1 gene as selectable marker placed under either the Thalassiosira pseudonana ACCase promoter, Nitzschia alba rpl41 promoter, or SV40 promoter. An available vacuole fcp promoter was also evaluated. Successful transformation was achieved in T. pseudonana, T. oceanica, and T. weissflogii using the fcp promoter, with T. pseudonana using the ACCase and rpl41 promoters, and N. alba using the rpl41 promoter. A protocol for the enrichment of protoplasts from N. alba was developed, as well as a method to generate auxospores from T. pseudonana. Constructs were made to test for homologous gene replacement in T. pseudonana. A direct-selection RNAi vector was constructed using an inverted repeat from the T. pseudonana tryptophan synthase α subunit fused to nitrate reductase expression control. Initial attempts at transformation and selection were encouraging with phenotypic results consistent with functional DNAi.					
15. SUBJECT TERMS Diatom, genetic manipulation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 13	19a. NAME OF RESPONSIBLE PERSON Mark Hildebrand
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (Include area code) 858 822-0167

Final Report
Development of New Genetic Manipulation Tools
For Metabolic Engineering of Diatoms
Mark Hildebrand, Scripps Institution of Oceanography, UCSD, La Jolla CA,
mhildebrand@ucsd.edu, (858) 822-0167

Abstract

This project's goal was to develop genetic manipulation tools for metabolic engineering of diatoms for biodiesel lipid production and other purposes. New diatom selectable markers were tested, relying on mutations to antibiotic resistance in two ribosomal protein genes. Three new diatom transformation vectors were constructed, using the *nat1* gene as selectable marker placed under control of either the *Thalassiosira pseudonana* ACCase promoter, *Nitzschia alba* *rpL41* promoter, or SV40 promoter. An available vector using the *fcp* promoter was also evaluated. Successful transformation was achieved in *T. pseudonana*, *T. oceanica*, and *T. weissflogii* with the *fcp* promoter, with *T. pseudonana* using the ACCase and *rpL41* promoters, and *N. alba* using the *rpL41* promoter. A procedure for the enrichment of protoplasts from *N. alba* was developed, as well as a method to generate auxospores from *T. pseudonana*. Constructs were made to test for homologous gene replacement in *T. pseudonana*. A direct-selection RNAi vector was constructed using an inverted repeat from the *T. pseudonana* tryptophan synthase β subunit fused to nitrate reductase expression control elements. Initial attempts at transformation and selection were encouraging, with phenotypic results consistent with functional RNAi. In summary, this project has developed several new tools for diatom genetic manipulation.

20090810265

I. Statement of Project Objectives

The overall goal of this project was to develop advanced genetic manipulation tools that could be used to facilitate metabolic engineering of diatoms for biodiesel lipid production and other purposes. The work focused on *Thalassiosira pseudonana*, the first diatom with a sequenced genome, but with an eye towards expansion into other relevant diatom species. Specific objectives were to:

1. Develop new selectable transformation markers that would enable sequential addition of engineered genes to facilitate multi-component metabolic engineering.
2. Develop "universal" transformation vectors that could be used across a broad range of diatom species without having to isolate species-specific promoters to drive selectable markers.
3. Develop new diatom transformation techniques including glass bead vortexing or the use of auxospores or protoplasts that would require less expensive equipment and provide higher transformation efficiencies.
4. Develop new gene manipulation tools, including inducible promoters, homologous gene replacement, and knockout approaches.
5. Develop new gene tagging approaches to facilitate determining protein-level expression control as well as intracellular targeting.

II. Results

II. A. Developing New Selectable Transformation Markers for Diatoms

Transformation of diatoms has been successfully achieved using the biolistic (gene gun) approach, where high velocity microparticles coated with DNA penetrate the cell wall¹⁻⁵. Selectable marker genes used for diatom transformation have included the neomycin phosphotransferase II (*nptII*) gene conferring G418 resistance², the *sh ble* gene conferring resistance to zeocin^{1,3,4}, and more recently the *natI* gene encoding resistance to nourseothricin⁶. The *sh ble* gene has been most widely used, but recent work demonstrates that *natI* can be superior⁶, most likely because the resistance-conferring protein is an enzyme, requiring lower expression levels compared with the *sh ble* protein that binds to antibiotics stoichiometrically. In *Thalassiosira pseudonana*, only the *natI* gene gave sufficient transformants⁶, indicating a need for additional selectable markers to be used for metabolic engineering.

Mutations in several ribosomal proteins have been shown to induce resistance to antibiotics in unicellular eucaryotes. We showed that *T. pseudonana* was sensitive to low levels of the antibiotics anisomycin (0.15 µg/ml) and emetine (6 µg/ml). Ribosomal protein L41 has been shown in a variety of yeasts to be involved in resistance to anisomycin and cycloheximide^{7,8}. A conserved mutation from proline to glutamine at amino acid residue 56 has been shown to confer resistance, which was confirmed by reversion to sensitivity with the reverse change⁷. The *T. pseudonana rpL41* gene encodes a proline at this position (Fig. 1A), consistent with its sensitivity. In ribosomal protein S14, different amino acid mutations at the C-terminal portion of the protein have been shown to confer resistance to emetine and cryptopleurine in several eucaryotes^{9,10}, and a dominant selectable transformation vector using an appropriately mutated gene was generated for *Chlamydomonas reinhardtii*¹¹.

A.

Pichia stipitis MVNVPKTRKTYCKGKECRKHTQHVKVTQYKAGKASLFAQGGKRRYDRKQSGYGGQTKQIFHK 60
Yarrowia lipolytica MVNIPKTRNTYCKGKECRKHTQHVKVTQYKAGKASLYAQGGKRRYDRKQSGYGGQTKQIFHK 60
Candida tropicalis MVNIPKTRNTYCKGKGRKHTIHKVTQYKAGRASLFAQGGKRRYDRKQSGYGGQTKQVFHK 60
S. cerevisiae MVNVPKTRKTYCKGKTCRKHTQHVKVTQYKAGKASLFAQGGKRRYDRKQSGYGGQTKPVFHK 60
A. nidulans MVNVPKTRKTYCKGKECHKHTQHVKVTQYKAGKASLFAQGGKRRYDRKQSGYGGQTKPVFHK 60
Oryza sativa MVNVPKTKKTYCKNKECRKHTLHKVTQYKKGKDSLFAQGGKRRYDRKQSGYGGQTKPVFHK 60
Homo sapiens MVNVPKTRRTFCK--KCGKHQPHKVTQYKKGKDSLFAQGGKRRYDRKQSGYGGQTKPIFRK 58
P. tricornutum MVNVPKERKTYCAG-KCKKHAHKVTQYKAGKASNFAQGGKRRYDSKQMGFGGQTKPVFHK 59
T. pseudonana MVNVPKERKTYCAGKCKRKHQVHKVTQYKAGKASNFAQGGKRRYDSKQMGFGGQTKPVFHK 60
 ***:* .:* *

Pichia stipitis KAKTTKKVVLKLECVV--CKAKSQLPLKRCCKHFELGGDKKQKGQALQF 106
Yarrowia lipolytica KAKTTKKVVLKLECVK--CKVKMLALKRCCKHFELGGDKKQKGQALQF 106
Candida tropicalis KAKTTKKIVLKLECTV--CKTKKQLPLKRCCKHIELGGEKKQKGQALQF 106
S. cerevisiae KAKTTKKVVLKLECVK--CKTRAQLTLKRCCKHFELGGEKKQKGQALQF 106
A. nidulans KAKTTKKVVLKLECTQ--CKTKKQLALKRCCKHFELGGDKKTKGAALVF 106
Oryza sativa KAKTTKKIVLKLCQS--CKHYSQHPKRCCKHFEIGGDKKKGKTSLF- 105
Homo sapiens KAKTTKKIVLRLCEVPCNCRSKRMLAIKRCCKHFELGGDKKRRKGQVIQF 106
P. tricornutum KAKTTKKVVLKLECKK--CKTKKQLCIRKTHFELGEKAKQSG---- 100
T. pseudonana KAKTTKKIVLRLCKT--CKQKKHLTLKRTKHFELGAKKAESGHQY-104
 *****:***** *: : ** ** ** **

B.

C. reinhardtii CRY1 DSTRKGGRRGRRL
C. reinhardtii CRY1-1 DSTRKGGRRGPRL
C. griseus (CHO) wild-type DSTRKGGRRGRRL
C. griseus (CHO) Emr-2 DSTRKGGRRGCRL
C. griseus (CHO) Emr-2-3 DSTRKGGRRGCHL
S. cerevisiae CRY1, CRY2 DSTRKGGRRGRRL
S. cerevisiae cry1 (31) DSTRKGGRRGRRL
S. cerevisiae cry2 (31) DSTRKGGRRGRRL
P. tricornutum DSTRKSGRRGRRL
T. pseudonana DSTRKSGRRGRRL

Figure 1. Site-directed mutagenesis of ribosomal proteins in *T. pseudonana* A. Amino acid alignment of the entire sequence of rpl41 from a variety of species listed at left. Number at right denotes the position of the last amino acid on the line. Below the alignments, (*) represents an identical amino acid in all species, (:) represents a highly conserved amino acid, (.) represents an acceptable substitution. The red amino acid at position 56 (arrow) locates the position of change from sensitivity to anisomycin (proline) to resistance (glutamine). B. Amino acid alignment of only the C-terminus of rpS14 from species identified at left. Red amino acids are those involved in conversion of the protein from sensitivity (e.g. *C. reinhardtii* CRY1) to resistance to emetine. Arrow indicates the location of the amino acid change in *T. pseudonana*.

We isolated *rpl41* and *rpS14* from *T. pseudonana* using the genome sequence as a guide to generate oligonucleotide primers for PCR, and cloned these genes under the control of the *T. pseudonana* *fcp* promoter, which drives high level expression of genes in this diatom⁶. Site-directed mutagenesis was performed to alter the specific amino acids shown to confer resistance to anisomycin (for L41) or emetine (for S14). For *rpl41*, the proline at position 56 (Fig. 1A) was altered to a glutamine, and for *rpS14*, we altered the arginine third from the end to a cysteine (Fig. 1B). *T. pseudonana* was transformed with these constructs, and selected appropriately. Resistant colonies were picked, and passaged through one more round of selection in liquid medium, then DNA was isolated from the putative transformants, and the presence of the mutated ribosomal protein gene

evaluated by PCR and sequencing. For the *rpL41* construct, one resistant colony was shown to contain the mutated gene, however, growth was slow during antibiotic selection, and growth also occurred in non-transformed cells. For the *rpS14* construct, growth was also slow, and non-transformed cells also grew, and no colonies were shown to contain the mutated gene.

In summary, the isolation of a single *rpL41* transformant containing the altered gene was encouraging; however, the general slow growth with both selection schemes was consistent with the lack of sufficient expression of the introduced mutant genes to overcome the effect of having copies of the native (non-mutated) gene in the cell. Under these conditions, the native and mutant genes will compete for incorporation into the ribosome, and if the percentage of ribosomes incorporating the mutant gene is not sufficient, then the resistance phenotype is marginal. This problem was encountered in developing a cycloheximide resistance marker in *C. reinhardtii*, and in that system overexpression with a stronger promoter overcame the problem⁷. For *T. pseudonana*, we have determined (see below) that the *fcp* promoter is one of the strongest ones available, but is not as strong as the *rpL41* promoter; therefore overexpression using a stronger promoter is not a good option for the diatom. Development of homologous gene replacement approaches (below) could enable complete replacement of the native gene, and provide a viable approach.

II.B. Developing Diatom Transformation Vectors That Can Be Used in Diverse Species.

Most diatom transformation vectors have used a promoter from the same species to be transformed (typically from the highly expressed but light-regulated fucoxanthin chlorophyll binding protein gene *fcp*). It is time-consuming to isolate an appropriate promoter sequence from the species to be transformed, and would be vastly more convenient if a transformation vector was developed with a promoter that was functional in most or at least multiple diatom species. Surprisingly, it has been overlooked that the initial diatom transformation work used a promoter from the *Cyclotella cryptica* acetyl-CoA carboxylase (ACCase) gene, which worked both in this species and in the evolutionarily distant species *Navicula saprophila*². This prompted us to test the ability of a variety of promoters to drive selectable marker gene expression in different diatom species.

We determined mRNA accumulation levels of genes encoding nitrate reductase (NR), ACCase, *fcp*, and *rpL41* using quantitative real-time PCR (qRT PCR), which indicated a range of promoter strengths ranging from a low with NR to a high with *rpL41* (Fig. 2). The fact that we have identified intermediate strength promoters in the ACCase and *fcp* genes will be useful because intermediate-level protein expression is sometimes desirable. We generated constructs using the *nat1* gene as a selectable marker under the control of 5' and 3' regions of the *rpL41* gene from *Nitzschia alba*, and the ACCase gene from *T. pseudonana*, and also using the viral SV40 5' region and *T. pseudonana* ACCase 3' region (Fig. 3).

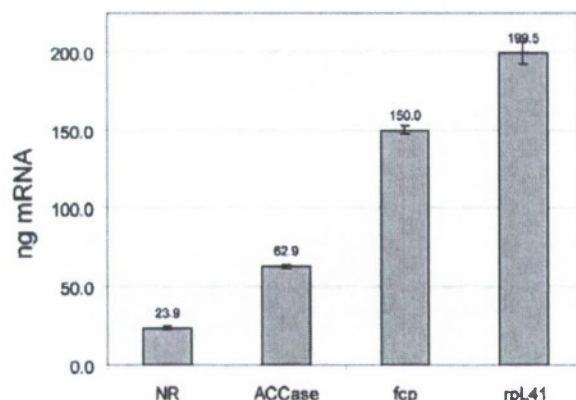


Figure 2. Measurement of mRNA accumulation levels for different promoters from *T. pseudonana*. RNA was extracted from exponentially-growing *T. pseudonana* and mRNA levels were determined for each gene using quantitative real-time PCR. Abbreviations: NR (nitrate reductase), ACCase (Acetyl CoA-carboxylase), fcp (fucoxanthin chlorophyll binding protein), rpl41 (ribosomal protein L41).

Using the *fcpNAT* transformation vector (provided by Nicole Poulsen) containing a *T. pseudonana fcp* promoter⁶, we have successfully transformed *T. pseudonana*, *T. oceanica*, and *T. weissflogii*. The *rpl41/nat* and *ACCase/nat* constructs have apparently successfully transformed *N. alba* and *T. pseudonana* (analysis is still ongoing). These two diatom species are evolutionarily distant, therefore successful transformation of each with two different promoters is highly encouraging that these vectors will work in multiple diatom species. We plan to continue testing the ability of these vectors to transform additional species.

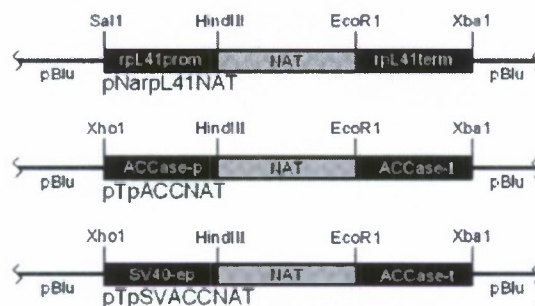


Figure 3. Maps of diatom transformation vectors. Three vectors are shown, pNarpL41NAT, pTpACCNAT, and pTpSVACCNAT. All vectors use the NAT gene as selectable marker, and are constructed in pBluescript (pBlu) plasmids. Restriction sites used for cloning are shown above each construct. pNarpL41NAT uses the promoter and terminator regions from the *rpl41* gene from *Nitzschia alba*. pTpACCNAT uses promoter and terminator regions from the *T. pseudonana* ACCase gene. pTpSVACCNAT uses the SV40 promoter and *T. pseudonana* ACCase terminator.

II.C. Developing New Diatom Transformation Techniques

The diatom silica cell wall presents a physical barrier to introduction of foreign material, but transformation has been successfully achieved using the biolistic (gene gun) approach, where high velocity microparticles coated with DNA penetrate the wall¹⁻⁵. Biolistic transformation is a low efficiency process, which prevents application of approaches such as genetic complementation of mutants by introduction of a genomic library to be applied to diatoms. For this reason we proposed to investigate novel approaches to transform diatoms, including glass bead vortexing, generation of auxospores, and generation of protoplasts. Glass bead vortexing involves vortexing cells in the presence of glass beads, DNA, and PEG-8000, and works successfully in *Chlamydomonas*¹². Since diatom cells can be broken open with glass bead vortexing, we decided to test whether this method could be used for transformation. Initial results were not promising, however this may be as much a function of our vortex apparatus as the diatom cell wall structure. We plan to further investigate this approach in the future.

Most diatoms prefer to divide vegetatively, but under certain conditions, can be induced to proceed through the sexual cycle, and after fusion of gametes, generate auxospores, which are large spherical cells covered in thin silicified scales¹³. Auxospores then produce vegetative cells. The swollen nature of auxospores (Fig. 4), coupled with their thin covering, suggests both that the plasma membrane is accessible and the silicified barrier would be more penetrable than in a vegetative cell. We (Davis and Hildebrand, unpublished data) developed methods to induce auxospores in *T. pseudonana* that involves prolonged treatment with copper or germanic acid (Fig. 4), but have not yet attempted transformation using them.

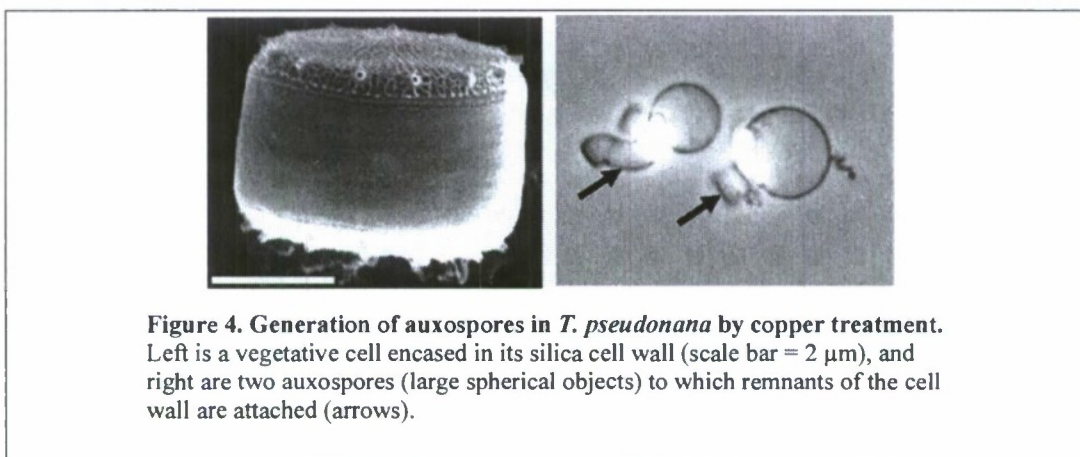


Figure 4. Generation of auxospores in *T. pseudonana* by copper treatment. Left is a vegetative cell encased in its silica cell wall (scale bar = 2 μ m), and right are two auxospores (large spherical objects) to which remnants of the cell wall are attached (arrows).

Nitzschia alba has a propensity to naturally form protoplasts^{14, 15}, in which the cytoplasm is entirely released from the silica shell, forming a spherical cell with no wall (Fig. 5). We have refined methods for protoplast generation and shown that the protoplasts are quite stable and can be enriched using a gradient centrifugation approach¹⁶. In addition, we have shown that the protoplast can regenerate a silicified cell wall. These results open the door towards using electroporation¹⁷ or chemical transformation procedures such as the use of lipophilic reagents¹⁸ or partially degraded cationic polyamidoamines (PAMAM's)¹⁹.

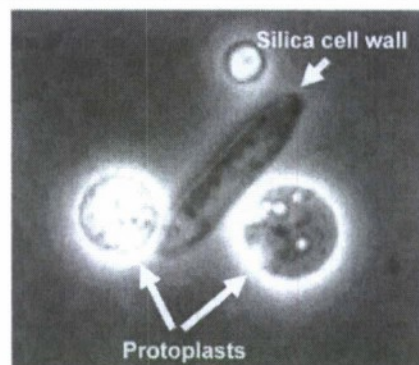


Figure 5. The silica cell wall and protoplasts of *Nitzschia alba*. Cell wall is approximately 16 μm in length.

II.D. Developing New Gene Manipulation Tools

Metabolic engineering of diatom cells for lipid production will require controlling the expression of specific introduced genes. Recently, the first control over expression of an introduced gene in diatoms was reported, using sequences from the nitrate reductase (*NR*) gene^{6,20}. In the presence of nitrate in the growth medium, mRNA and protein of genes placed under the control of *NR* are induced, in the absence of nitrogen, only mRNA is induced and no protein is made, and in ammonium, mRNA and protein are repressed^{6,20}. There is also some control over the level of induction in the presence of nitrate²⁰.

To date, DNA introduced into diatoms has recombined randomly into the genome with varying numbers of integrated copies¹⁻⁶, and homologous gene replacement through recombination has not been reported. The presence of both modified and native genes in diatom transformants could create problems for metabolic engineering, because if both gene products are expressed, there could be phenotypic competition. Homologous gene replacement would completely eliminate the native gene's phenotype, and is desirable for metabolic engineering. In addition, homologous replacement of a gene with a non-functional copy would enable gene knockouts.

We explored homologous gene replacement and knockout generation choosing a redundant *fcp* gene (there are multiple *fcp* genes in the *T. pseudonana* genome, suggesting overlap in function) and inserting a termination codon and restriction site near the amino terminus. The termination codon should knockout synthesis of functional protein, and the restriction site provides a means to monitor by PCR whether the introduced gene has replaced the native one in the same chromosomal location. After biolistic transformation, we can evaluate genomic insertion of the modified gene using one PCR primer whose 3'-end has the introduced termination codon and restriction site (unique to the modified gene) and another in the flanking region. We can then evaluate whether the insertion has occurred in the native chromosomal location using a PCR primer outside of the cloned fragment – correct localization will result in a PCR product whereas incorrect will not. Our initial attempt was done using double stranded plasmid DNA. Transformants were isolated that had the introduced modified *fcp* gene, however, the gene did not replace the one in the native location. It has been shown that single-

stranded DNA significantly reduces random genomic insertion and favors homologous replacement in *Chlamydomonas* and other organisms^{21,22}; therefore in our second attempt, we generated single stranded DNA from our *fcp* construct by linear amplification, and introduced that into *T. pseudonana*. Transformants are currently being analyzed to determine whether the modified gene has replaced the native gene.

RNA interference (RNAi) is a useful method for inhibiting gene expression²³, and we proposed to develop RNAi approaches for diatoms. RNAi machinery in other organisms involves the Dicer protein, which recognizes double stranded RNA and cleaves it into ~ 22 bp fragments that become bound by the RISC or RITS protein complexes, which then carry out specific transcriptional silencing or transcript degradation²³. Although Dicer genes were not clearly identified in the initial genome analyses²⁴, a more rigorous bioinformatics analysis by graduate student Trina Norden-Krichmar identified Dicer gene homologs with similarity to some plant Dicers. This encouraged attempts to develop RNAi.

A positive selection approach for RNAi has been developed in *Chlamydomonas reinhardtii* by generating a construct with inverted repeats of both the target gene and the *Maa7* gene encoding the tryptophan synthase β subunit, which when inactivated provides resistance to the tryptophan analogue 5-fluoroindole²⁵. This strategy correlates silencing with the level of resistance to 5-fluoroindole²⁵. We constructed (Davis and Hildebrand, unpublished data) a similarly designed vector, using sequences from the *T. pseudonana* tryptophan synthase β subunit gene, placed under control of the NR promoter to allow control of silencing. Biolistic transformation was done, and cells were incubated under two conditions prior to replating on the selection medium, one condition was in the presence of nitrate, and the other in ammonium. Because expression of the inverted repeat was under control of the NR promoter, one would expect expression in nitrate and repression in ammonium. Cells were incubated 24 hr, then cells from each treatment were plated on different concentrations of 5-fluoroindole (5-FI) in the presence of tryptophan (to compensate for the possible lack of a functional native tryptophan synthase β subunit) in agar medium containing either nitrate or ammonium as nitrogen source. The results of the different pretreatment and plating regimes are shown in Fig. 6. No growth was observed on ammonium-containing plates. Cells pretreated with ammonium produced 9 colonies on 4 μ M 5-FI plates, whereas pretreatment with nitrate produced 128 colonies (Fig. 6). These results are consistent with activation of RNAi by pretreatment with nitrate and with nitrate-containing plates. Transformants are currently being selected onto fresh plates. After growth, the presence of the introduced construct will be tested by PCR, and RNAi tested by examining the intactness of mRNA for the tryptophan synthase β subunit in the presence of ammonium (expected to be intact) and nitrate (expected to be degraded). These results are highly encouraging that direct selection RNAi is functional in diatoms.

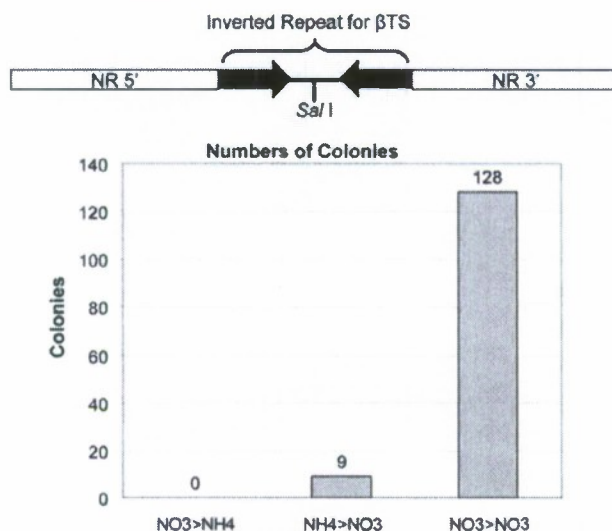


Figure 6. Direct selection RNA interference (RNAi) vector and results for *T. pseudonana*. Upper, diagram of direct selection RNAi vector. NR is the 5' and 3' portions of the nitrate reductase gene to control expression. βTS is an inverted repeat designed from the tryptophan synthase β subunit gene that generates RNAi. *SalI* is a restriction site. Lower, numbers of colonies resulting from pretreatment with either NO₃ or NH₄, plated on plates containing either NO₃ or NH₄

II.E. Developing New Gene Tagging Approaches

Gene tagging approaches using fusions to green fluorescent protein (GFP) have been successfully applied to diatoms, enabling determination of intracellular protein location and protein expression levels in some species^{3, 6, 9, 26}. Recent work in our lab and that of our collaborators Nicole Poulsen and Nils Kröger (Georgia Tech) using a GFP-based reporter system in *Thalassiosira pseudonana* has shown mixed results; cell wall targeted proteins can work well²⁷, but with a variety of tagged intracellular proteins GFP protein is present (detected via antibody) but fluorescence absent. The reasons for this are not entirely clear; we suspect that it could be due to improper GFP folding, improper cellular localization, or perhaps oxygen deprivation due to the small cell size of *T. pseudonana*. We had planned to investigate the applicability of the TC tag to fluorescently track diatom proteins. TC tags are 20 amino acid peptides fused to the protein of interest containing 4 specifically spaced cysteine residues that bind biarsenical dyes²⁸. Two dyes are available, FIAsh or ReAsh, which fluoresce green or red respectively upon binding to the cysteines. Discussion with the developers of this tagging system indicated that it was excessively expensive, and they only relied on it after extensive experimentation with GFP tagged protein. We therefore abandoned this approach. We have recently begun constructs using epitope tags, specifically the V5 epitope. Localizing proteins by epitope tagging is a well established approach²⁹.

III. Summary of Project Results

Site-directed mutagenesis was used to introduce amino acid changes to the sequences of the *T. pseudonana* *rpS14* and *N. alba* *rpL41* genes to confer resistance to emetine and anisomycin, respectively. Transformation attempts were made in *T. pseudonana*, and resulted in one positive transformant using *rpL41*, however for both genes, slow growth and background growth with non-transformed cells occurred. Further development of this approach is likely to require homologous gene replacement to completely remove the effect of the native genes. Three new diatom transformation vectors were made, using the *nat1* gene as selectable marker placed under control of either the *T. pseudonana* ACCase promoter, *N. alba* *rpL41* promoter, or SV40 promoter. An available vector using the *fcg* promoter was also evaluated. Successful transformation was achieved in *T. pseudonana*, *T. oceanica*, and *T. weissflogii*, with the *fcg* promoter, with *T. pseudonana* using the ACCase and *rpL41* promoters, and *N. alba* using the *rpL41* promoter. A procedure for the enrichment of protoplasts from *N. alba* was developed, as well as a method to generate auxospores from *T. pseudonana*, but no transformation attempts using protoplasts or auxospores were made. Constructs were made to test for homologous gene replacement in *T. pseudonana* by introducing a termination codon and restriction site in an *fcg* gene. Initial attempts indicated genomic incorporation but not homologous replacement; current attempts are utilizing single-stranded DNA to favor homologous recombination. A direct selection RNAi vector was constructed using an inverted repeat from the *T. pseudonana* tryptophan synthase β subunit fused to nitrate reductase expression control elements. Initial attempts at transformation and selection were highly encouraging, with phenotypic results consistent with functional RNAi. Further characterization is ongoing.

In summary, this project has developed several new tools for diatom genetic manipulation which will aid in the metabolic engineering of cells for biofuel lipid production and other purposes.

IV. Personnel Supported

Dr. Mark Hildebrand – Research Scientist
Dr. Luciano Frigeri – Staff Research Associate
Mr. Jeffrey Carlson – M.S. student

V. Publications

None to date. Data generated from this one-year project will be included in future papers.

VI. Student Theses

Jeffrey Carlson, M.S. Thesis, "Microscopic Studies in Nascent Frustule Formation from Protoplasts of the Marine Pennate Apochlorotic Diatom *Nitzschia alba* and Production of Novel Transformation Vectors for Studies in *N. alba* and the Marine Centric Photoautotrophic Diatom *Thalassiosira pseudonana*."

available at:

<http://proquest.umi.com/pqdlink?Ver=1&Exp=08-21-2013&FMT=7&DID=1507553341&RQT=309&attempt=1&cfc=1>

Summary of Thesis

Site-directed mutagenesis was done to convert the *T. pseudonana* *rpS14* and *Nitzschia alba* *rpL41* genes into mutants capable of conferring resistance to emetine and anisomycin, as described in section II.A. For *N. alba*, this required isolation of the *rpL41* gene using degenerate oligonucleotide primers, coupled with inverse PCR to obtain the full-length gene sequence. For *T. pseudonana*, the genome sequence was used to generate primers for cloning. The vectors described in section II.D. and Fig. 3 were generated for transformation of *N. alba* and *T. pseudonana*. The vectors were used in attempts to transform these two diatom species, with results as described in section II.D. Although antibiotic resistance colonies were obtained, it was not convincingly confirmed that the constructs had incorporated into the genome. Based on previous work (), protoplasts of *N. alba* were generated, and a gradient centrifugation purification protocol developed to enable their enrichment. Protoplasts were shown to regenerate their cell walls. No attempts to transform protoplasts were made.

VII. Interactions/Transitions

M. Hildebrand, invited speaker at AFOSR NREL Algal Biofuels Workshop, "Development of Algal Genetic Tools" Arlington, VA Feb. 19-21, 2008.

VIII. New Discoveries, inventions, or patent disclosures

None.

IX. Honors/Awards

None.

References

1. Apt K.E., Kroth-Pancic P.G., and Grossman A.R., *Mol. Gen. Genet.*, 1996, **252**: 572.
2. Dunahay T.G., Jarvis E.E., and Roessler P.G., *J. Phycol.*, 1995, **31**: 1004.
3. Falciatore A., et al., *Mar. Biotech.*, 1999, **1**: 239.
4. Fischer, H., et al., *J. Phycol.*, 1999, **35**: 113.
5. Zaslavskaja L.A., et al., *J. Phycol.*, 2000, **36**: 379.
6. Poulsen, N., P.M. Chesley, and N. Kroger, *J. Phycol.*, 2006, **42**: 1059.
7. Del Pozo, L., et al., *Eur. J. Biochem.*, 1993, **213**: 849.
8. Dehoux, P., J. Davies, and M. Cannon, *Eur. J. Biochem.*, 1993, **213**: 841.
9. Larkin, J.C., J.R. Thompson, and J.L. Woolford, *Mol. Cell. Biol.*, 1987, **7**: 1764.
10. Rhoads, D.D. and D.J. Roufa, *Mol. Cell. Biol.*, 1985, **5**: 1655.
11. Nelson, J.A.E., P.B. Saveriede, and P.A. Lefebvre, *Mol. Cell. Biol.*, 1994, **14**: 4011.
12. Nelson, J.A.E. and P.A. Lefebvre, *Mol. Cell. Biol.*, 1995, **15**: 5762.
13. Round F.E., Crawford R.M., and Mann D.G., *The diatoms: Biology and morphology of the genera*. 1990, Bath: Cambridge University Press. pp.747.
14. Bhattacharyya, P. and B.E. Volcani, *Proc. Natl. Acad. Sci. USA*, 1981, **77**: 6386.
15. Hemmingsen, B.B., *A mono-silicic acid stimulated adenosinetriphosphatase from protoplasts of the apochlorotic diatom Nitzschia alba*, Ph.D. Thesis. 1971, UCSD: La Jolla CA. pp. 119.
16. Carlson, J., "Microscopic Studies in Nascent Frustule Formation from Protoplasts of the Marine Pennate Apochlorotic Diatom *Nitzschia alba* and Production of Novel Transformation Vectors for Studies in *N. alba* and the Marine Centric Photoautotrophic Diatom *Thalassiosira pseudonana*." M.S. Thesis, 2008, University of California, San Diego, La Jolla CA, pp. 127.
17. Gehl, J. and L.M. Mir, *Biochem. Biophys. Res. Commun.*, 1999, **261**: 377.
18. Felgner, P.L., et al., *Proc. Natl. Acad. Sci. USA*, 1987, **84**: 7413.
19. Bielinska, A., et al., *Nucl. Acids Res.*, 1996, **24**: 2176.

20. Poulsen N. and Kröger N., *FEBS J.*, 2005, **272**: 3413.
21. Zorin, B., P. Hegemann, and I. Sizova, *Euc. Cell*, 2005, **4**: 1264.
22. Simon, J.R. and P.D. Moore, *Mol. Cell. Biol.*, 1987, **7**: 2329.
23. Hannon, G.J., *Nature*, 2002. **418**: 244.
24. Armbrust, E., J. Berges, et al. *Science* 2004, **306**:79.
25. J. Rohr, N. Sarkar, S. Balenger, B. Jeong and H. Cerutti, *The Plant Journal* 2004, **40**: 611.
26. Apt, K.E., et al., *J. Cell Sci.*, 2002, **115**: 4061.
27. Poulsen, N., et al., *Angew. Chemie*, 2007, **46**: 1843.
28. Gaietta, G., et al., *Science*, 2002, **296**: 503.
29. J. W. Jarvik and C. A. Telmer, *Ann. Rev. Genet.* 1998, **32**: 601.

AIR FORCE OFFICE OF SCIENTIFIC RESEARCH

16 MAR 2009

DTIC Data

Page 1 of 2

Purchase Request Number: FQ8671-0701052
BPN: F1ATA07040B002
Proposal Number: 07-NL-114
Research Title: DEVELOPMENT OF NEW GENETIC MANIPULATION TOOLS FOR METABOLIC ENGINEERING OF DIATOMS
Type Submission: New Work Effort
Inst. Control Number: FA9550-07-1-0386DEF
Institution: SCRIPPS INSTITUTION OF OCEANOGRAPHY
Primary Investigator: Dr. Mark Hildebrand
Invention Ind: none
Project/Task: 2312A / X
Program Manager: Walter Kozumbo

Objective:

: The objective of this project is to develop advanced genetic manipulation tools for diatoms to enable metabolic engineering for biodiesel production and other purposes. The work will focus on *Thalassiosira pseudonana*, the first diatom with a sequenced genome, with an eye towards expansion into other relevant diatom species.

Approach:

Various new molecular biological tools will be developed to enable the metabolic engineering to proceed smoothly. First, selectable diatom transformation markers will be developed for the sequential addition of engineered genes; they will facilitate multi-component metabolic engineering by using highly conserved mutations in ribosomal proteins for antibiotic resistance. Second, "universal" transformation vectors will be developed that could be used across a broad range of diatom species, using viral promoters such as CaMV 35S, SV40, or CMV IE1. Third, new diatom transformation techniques will be developed, including glass bead vortexing or the use of auxospores or protoplasts that would require less expensive equipment and provide higher transformation efficiencies. Electroporation or surfactant- or polyamine-based chemical procedures will be applied to introduce DNA into cells that lack the usual silicified cell wall. Fourth, new gene manipulation tools will be developed, including inducible promoters, homologous gene replacement, and knockout approaches, will be used. Copper-inducible gene promoters, which were previously identified by us, will be used to control expression of introduced genes. We intend to develop homologous gene replacement approaches will be developed by introducing large flanking regions of the gene of interest and/or single stranded DNA. Gene knockout approaches using homologous gene replacement or RNA interference (RNAi) will also be explored. Finally, new gene tagging approaches (TC and SNAP tags) will be developed to facilitate determining protein-level expression control as well as intracellular targeting.

Progress:

Year: 2007 **Month:** 02

Not required at this time.

Year: 2008 **Month:** 01

We have made constructs and are testing two new selectable marker genes for diatom transformation using mutants in two ribosomal protein genes, rpS14 (emetine resistance) and rpL14 (anisomycin resistance). Using the nourseothricin resistance (nat) gene combined with either the ACCase, SV40, or rpL41 promoters, we have developed vectors capable of transforming four diatom species, *Thalassiosira pseudonana*, *T. weissflogii*, *T. oceanica*, and *Nitzschia alba*. These results suggest universal diatom transformation vectors that can be used in multiple species are feasible. We have made

AIR FORCE OFFICE OF SCIENTIFIC RESEARCH

16 MAR 2009

DTIC Data

Page 2 of 2

Progress:

Year: 2008 Month: 01

constructs to test homologous gene replacement by introducing a termination codon and restriction site in a *T. pseudonana* fcp gene. We also have made a construct using portions of the beta-tryptophan synthase gene from *T. pseudonana* for RNA interference (RNAi), based on a similar system developed in *Chlamydomonas reinhardtii*. This system enables positive selection for RNAi by growth on 5-fluoroindole, which we have shown *T. pseudonana* is sensitive to. We have completed a construct using the V5 epitope sequence for epitope tag localization of proteins inside *T. pseudonana*, to be tested by generating fusions with genes of interest.

Year: 2008 Month: 08 Final

This project's goal was to develop genetic manipulation tools for metabolic engineering of diatoms for biodiesel lipid production and other purposes. New diatom selectable markers were tested, relying on mutations to antibiotic resistance in two ribosomal protein genes. Three new diatom transformation vectors were constructed, using the *nat1* gene as selectable marker placed under control of either the *Thalassiosira pseudonana* ACCase promoter, *Nitzschia alba* rpl41 promoter, or SV40 promoter. An available vector using the fcp promoter was also evaluated. Successful transformation was achieved in *T. pseudonana*, *T. oceanica*, and *T. weissflogii* with the fcp promoter, with *T. pseudonana* using the ACCase and rpl41 promoters, and *N. alba* using the rpl41 promoter. A procedure for the enrichment of protoplasts from *N. alba* was developed, as well as a method to generate auxospores from *T. pseudonana*. Constructs were made to test for homologous gene replacement in *T. pseudonana*. A direct-selection RNAi vector was constructed using an inverted repeat from the *T. pseudonana* tryptophan synthase β subunit fused to nitrate reductase expression control elements. Initial attempts at transformation and selection were encouraging, with phenotypic results consistent with functional RNAi. In summary, this project has developed several new tools for diatom genetic manipulation.